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Determination of the therapeutic effects of apigenin on chronic myeloid leukemia stem cells: a mechanistic approach

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ABSTRACT

Aims: Chronic myeloid leukemia (CML) is a type of leukemia characterized by the Philadelphia (Ph) chromosome encoding the BCR-ABL protein. Significant success has been achieved in the treatment of CML with the development of agents that target this protein and inhibit its activity, but the resistance that develops in patients against these drugs limits absolute success. One of the main reasons for the resistance problem is the ineffectiveness of current therapies for CML stem cells. Apigenin (4',5,7-trihydroxyflavone) is a flavanoid found in various vegetables and fruits and has the effects of suppressing proliferation and inducing apoptosis in different cancer types. In this study, we aimed to determine the therapeutic potential of apigenin on K562 CML stem cells.

Methods: The effects of apigenin on the proliferation of K562 CML stem cells were determined by an XTT cell proliferation assay. Apoptotic effects of apigenin on K562 CML stem cells were determined by changes in mitochondrial membrane potential (MMP), caspase-3 enzyme activity, and the Annexin-V method using flow cytometry.

Results: The proliferation of K562 CML stem cells exposed to increasing doses of apigenin (1-40 nM) for 48 hours decreased dose-dependently, and the IC50 value of Apigenin was calculated at 3 nM. Compared to the control group, an increase in caspase-3 enzyme activity was calculated in stem cells treated with apigenin at the same doses. Disruptions in the mitochondrial membrane potential of apigenin-treated CML stem cells and Annexin-V assay results also showed that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population.

Conclusion: It was shown for the first time in this study that apigenin may have therapeutic effects on CML stem cells. It was determined that this effect of apigenin was achieved by triggering caspase-3 enzyme activity and disruptions in the mitochondrial membrane.

Keywords: Apigenin, chronic myeloid leukemia stem cells, apoptosis, cytotoxicity

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal malignant disease of bone marrow stem cells with a specific chromosomal defect and is characterized by a specific chromosomal abnormality called the Philadelphia (Ph) chromosome. The Ph chromosome is formed by translocation of the BCR and ABL genes located on chromosomes 9 and 22, and the hybrid gene formed as a result of this translocation encodes a protein called BCR/ABL with tyrosine kinase activity that activates signaling pathways that enable uncontrolled growth and division of the cell.[1,2] Although great success has been achieved in the fight against the disease with tyrosine kinase inhibitors targeting BCR/ABL activity, the development of drug resistance is seen as the most important problem encountered in the clinic against these drugs.[3-5] For this reason, many studies are being conducted to overcome the resistance problem that results in the accumulation of primitive cells in the blood and bone marrow. However, in recent years, studies have been developed to develop therapies targeting CML stem cells for the treatment of CML.[6]

Cancer stem cells (CSCs) are known as a subpopulation of small cancer cells with stem cell-like properties and the

ability to produce all cancer cells.[7] Leukemic stem cells (LSCs) play a crucial role in disease incidence, drug resistance, and relapse.[8] LCSCs are regulated by critical surface antigens such as CD34 and CD38 proteins. These proteins are expressed in most cases of leukemia and are therefore used as specific cell markers in both the diagnosis and prognosis of the disease.[9,10] Therefore, targeting these cells will be a beacon of hope in cancer therapy.

Apigenin,(5,7-dihydroxy-2-[4-hydroxypheny]-4H-1-benzopyran-4-one) is a flavone commonly found in onions, grapefruit, oranges, parsley, sand chamomile.[11] Apigenin has an important place in the literature with its potential to be used in cancer prevention and treatment due to its activity in suppressing cell growth in different human cancer cell lines such as leukemia, thyroid, skin, prostate, colon, and breast cancer.[12] This flavone inhibits cancer cell proliferation by triggering cell apoptosis, inducing autophagy, and modulating the cell cycle. Apigenin also reduces cancer cell motility and inhibits cancer cell migration and invasion. Recently, apigenin has been reported to exhibit anti-cancer activities by stimulating an immune response.[13-15] There are studies showing the anti-proliferative effects of apigenin on CML, but no study showing its effect on CML stem cells has been found yet. Targeting cancer stem cells in cancer treatment is promising for cancer patients, as it will reduce the risk of disease recurrence. This study aimed to demonstrate the antiproliferative effect of apigenin on CML stem cells.

METHODS

This study does not require an ethics committee approval.It was approved by Erciyes University Scientific ResearchProject Coordinatorship as a multi-disciplinary research project with project code TCD-2015-5427 All ethical principles were respected in this study. Chronic myeloid cancer stem cells (K562) obtained from the Department of Molecular Biology and Genetics at the İzmir Institute of Technology were used in this study. Flow cytometry results showed that 99.58% of the selected cells were CD38 negative and 94.21% were CD34 positive, indicating that these cells were CML stem cells. These cells were propagated in culture, and the experimental stages of the study were completed. Apigenin, the chemical used in our study, was purchased from Sigma Aldrich (USA). The necessary stock solutions for this chemical were prepared in solvents at the ratios specified by the manufacturer and kept under appropriate storage conditions.

XTT Cell Proliferation Test

An XTT cell proliferation assay was used to determine the cytotoxic effect of apigenin on CML stem cells. These cells were treated with apigenin for 72 hours. For this purpose, CML stem cells were seeded with 10,000 cells in 100 µl of medium in each well of a 96-well plate, and the cells were incubated for 72 hours at 37°C in an incubator containing 5% CO_2 by adding the relevant agents at increasing concentrations in volumes of 100 µl each. At the end of incubation, 20 µl of XTT reagent was added to each well and incubated for 4 hours. Then, the solution formed in 96-well plates was read at 450nm wavelength in a spectrophotometer. A cell proliferation graph was generated according to the spectrophotometric results. The IC50 and IC10 values of all these agents in CML stem cells (the drug dose at which the proliferation of cells is suppressed by 50% and 10% compared to the control group without drug administration) were calculated from the cell growth graph.[16]

Determination of the Apoptotic Effect of Apigenin in CML Stem Cells by Phosphatidylserine Location on the Cell Surface

K-562 cells were seeded with $1x10^6$ cells in 2 ml of medium in each well of a 6-well plate, and cells were treated with increasing concentrations of apigenin and kept in an incubator containing 5% CO_2 at 37°C for 48 and 72 hours. At the end of the incubation period, the cells were centrifuged at 1000 rpm for 10 min, and the cell pellet was homogenized with 1 ml of buffer solution (PBS), and centrifugation was repeated. After centrifugation, 200 µl of Annexin binding solution was added to the cell pellet and homogenized. Then, 2 µl Annexin V and 2 µl propidium iodide were added to this mixture and incubated for 15 minutes at room temperature in the dark. Measurements were then performed using flow cytometry.[17]

Determination of the Effects of Apigenin on Caspase-3 Enzyme Activity in CML Stem Cells

CML stem cells were seeded with $1x10^6$ cells in 2 ml medium in each well of a 6-well plate, and apigenin was added to these cells at increasing concentrations. The cells were kept in an incubator at 37°C with 5% CO_2 for 48 and 72 hours and then centrifuged at 1000 rpm for 10 min. At the end of centrifugation, the cell pellet was homogenized with 50 µl of cell lysis solution. These cells were then centrifuged at 10,000 rpm for 1 min and the supernatant was treated with 200 µl of cell lysis solution. Then, 50 µl of sample, 50 µl of reaction solution, and 5 µl of DEVD-pNA were added to a 96-well plate, two wells for each sample, incubated at 37°C in 5% CO_2 for 2 h, and measured under the 405 nm wavelength in a spectrophotometer. Changes in caspase-3 enzyme activity were determined by proportioning these values measured for each sample to total protein amounts.[17]

Determination of Changes in Mitochondrial Membrane Potential

CML stem cells were seeded with $1x10^6$ cells in 2 ml medium in each well of a 6-well plate, and cells were treated with apigenin alone and incubated at 37°C in an incubator containing 5% CO_2 for 48 hours. When the incubation period was completed, the cells were collected, transferred to separate falcon tubes, and centrifuged at 1000 rpm for 10 min. After centrifugation, the supernatant on the cell pellet was discarded, and the cell pellet was homogenized with 300 µl of medium. Then, 30 µl of JC-1 dye was added to these cells, and the cells were kept in a CO_2 incubator at 37°C for 30 minutes. After this time, the cells were centrifuged at 1000 rpm for 5 min, and then the supernatant was discarded and the cell pellet was homogenized with 200 µl of assay buffer solution. This step was repeated once more, and 320 µl of assay buffer solution was added to the cell pellet to homogenize the cells. Then, 100 µl of each sample was seeded in triplicate in a 96-well plate and measured under 485/560 nm wavelengths in aspectrophotometer.[16,17]

RESULTS

XTT Cell Proliferation Test Results

The proliferation of K562 CML stem cells exposed to increasing doses of apigenin (1-40 nM) for 48 hours decreased dose-dependently, and the IC50 value of apigenin was calculated at 3 nM. The proliferation percentage of K562 CML stem cells treated with 1-, 5-, and 10 nM apigenin decreased by 34%, 80%, and 96%, respectively, compared to the control group (Figure 1).

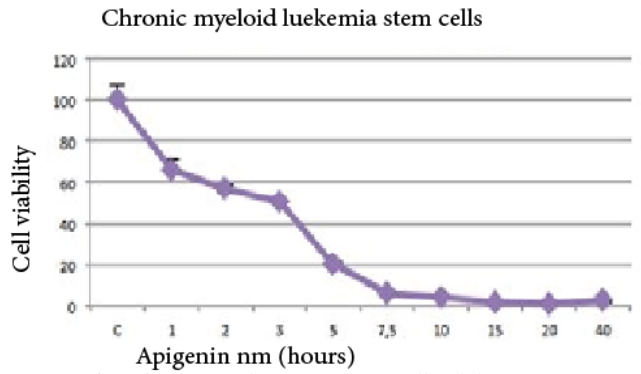

Figure 1. Effect of apigenin administration on cell viability in CML stem cells

Annexin V Test Results

This test is based on the principle that phosphatidylserine, which is found in the inner part of healthy cell membranes, moves to the outer part of the cell with the breakdown of the cell membrane in apoptotic cells. Using flow cytometry and the Annexin-V/Propidium Iodide double staining method, it was possible to determine the location and amount of phosphatidylserine. Annexin-V analysis results of apigenin-treated CML stem cells showed that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population (Figure 2).

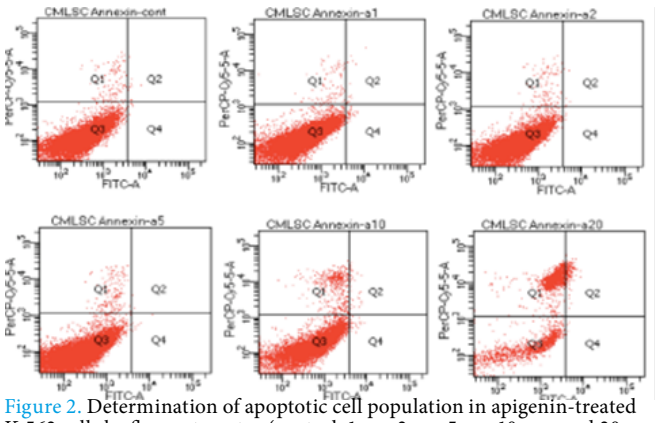

Figure 2. Determination of apoptotic cell population in apigenin-treated K-562 cells by flow cytometry (control, 1nm, 2nm, 5nm, 10 nm, and 20nm, respectively) Caspase-3 Enzyme Activity

Changes in caspase-3 enzyme activity of CML stem cells exposed to increasing doses of apigenin were determined by a caspase-3 colorimetric enzyme kit (BioVision Research Products, USA). This kit is based on the principle that the caspase-3 enzyme recognizes the substrate DEVD sequence and cleaves the DEVD-pNA complex, and as a result of this cleavage, the pNA that emits radiation is detected at a wavelength of 405 nm in a spectrophotometer. Compared to the control group, 8%, 53%, and 82% increases were calculated in caspase-3 enzyme activity of stem cells treated with apigenin at the same doses (Figure 3).

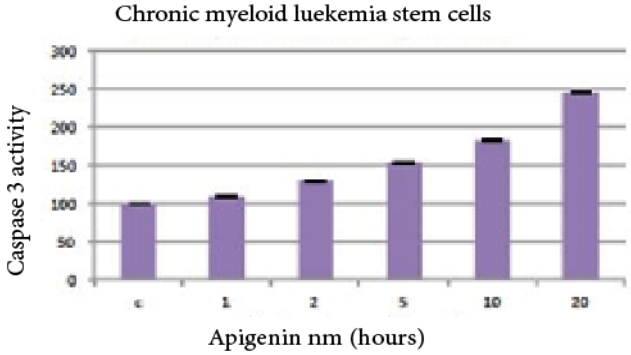

Figure 3. Changes in caspase-3 enzyme activity in K-562 cells treated with increasing doses of apigenin

These results supported that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population.

Mitochondrial Membrane Potential Test Results

The JC-1 mitochondrial membrane potential measurement kit (Cayman Chemicals, USA) measured changes in mitochondrial membrane potential in CML stem cells treated with apigenin for 48 hours to determine whether apoptosis had occurred. In light of cell proliferation data, CML stem cells were exposed to increasing doses of apigenin (1nm, 2nm, 5nm, 10 nm, and 20nm) for 48 hours, and changes in mitochondrial membrane degradation were determined. According to the results of the JC-1 assay, an increase of 5nm and above in the mitochondrial membrane potential degradation of CML stem cells exposed to increasing doses for 48 hours was detected. Disruptions in the mitochondrial membrane potential of CML stem cells treated with apigenin showed that apigenin triggered apoptosis in K-562 cells (Figure 4).

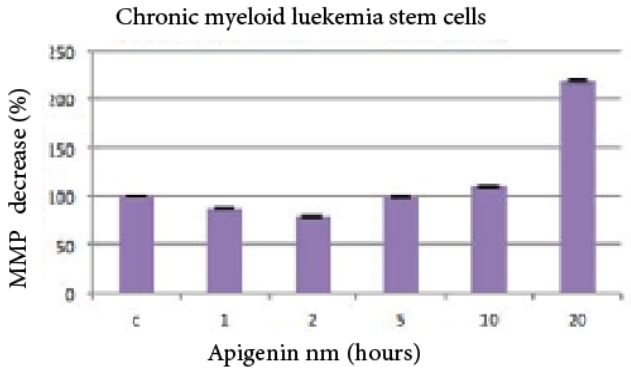

Figure 4. Changes in the mitochondrial membrane potential (MMP)s of K-562 cells with increasing concentrations of apigenin

DISCUSSION

(CML) is a type of leukemia characterized by the Philadelphia (Ph) chromosome encoding the 2010 kDa BCR-ABL (p210) fusion protein.[16] Although significant success has been achieved with the development of tyrosine kinase inhibitors (TKIs) that target the BCR-ABL protein and inhibit its activity and the application of these drugs in CML patients, the resistance that develops against these agents in patients limits the absolute success of CML treatment. One of the main reasons for the

problem of resistance is the lack of efficacy of these agents son CML stem cells.[17-19] apigenin (4',5,7- trihydroxyflavone) is a flavanoid found in different vegetables and fruits and has been shown to suppress cellular growth and trigger apoptosis in different cancer types.[20] In this study, we aimed to determine the therapeutic potential of apigenin on K562 CML stem cells.

Apoptosis is one of the main cell death mechanisms in response to cancer therapy.[21] Apoptosis is controlled by a complex series of interactions between pro-apoptotic proteins (Bax/Bak-like proteins and BH3-single protein) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL, Mcl-1) that facilitate apoptosis.[22] Bcl-2 inhibits mitochondrial permeability and subsequent cell death triggered by the pro-apoptotic Bax and Bak.[21,22] Most anti-cancer agents act by triggering the mitochondrial apoptotic pathway involving outer mitochondrial membrane permeability.[19] This process is controlled by pro- and anti-apoptotic members of the Bcl-2 family and causes cytosolic release of mitochondrial intermembrane proteins, including cytochrome c, leading to caspase activation.[19] In CML, treatment options targeting Bcl-2 and the caspase pathway have been shown to be beneficial by suppressing cell proliferation and increasing cell apoptosis.[23,25]

CONCLUSION

In this study, apigenin suppressed cell proliferation in CML stem cells. In addition, the increase in caspase-3 enzyme activity, disruptions in mitochondrial membrane potential, and annexin-V analysis results showed that apigenin dose-dependently triggered apoptosis and caused significant increases in the apoptotic cell population. We believe that these results will guide clinical studies, especially in patients who develop treatment resistance.

ETHICAL DECLARATIONS

Ethics Committee Approval

This study does not require an ethics committee approval. It was approved by Erciyes University Scientific Research Project Coordinatorship as a multi-disciplinary research project with project code TCD-2015-5427.

Informed Consent

Since this study was performed only on the cell line, consent was not obtained.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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